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EFFECTS OF SALINITY AND NUTRIENTS ON MONO- AND DIAXENIC CULTURES OF TWO STRAINS OF ARTEMIA SALINA[1]

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Bacteria-free cultures of crustaceans are needed to determine the nutritional value of different algae and to study the requirements of typical grazers.

Previous work was done with the bisexual Utah strain of *Artemia salina* (Great Salt Lake) (Provasoli *et al.*, 1959b). Wintering eggs were commercially available, easily washed free of bacteria, and easy to hatch. These obvious advantages facilitated development of monaxenic and axenic culture methods (Provasoli *et al.*, 1959a). However, the Utah strain had disadvantages: to obtain successive generations males and females had to be brought together to copulate, but copulation was not always successful and in most cases, wintering eggs were deposited. These eggs, for hatching, had to be collected, air-dried, and reincubated in a hatching medium. These aseptic manipulations enhanced risk of contamination. Detection of possible long-range nutritional deficiencies, which often appear only after several generations, would be too laborious. To by-pass these difficulties the parthenogenetic strain of *Artemia salina* from Comacchio was axenized.

Materials and Methods

Source

The Comacchio strain of *Artemia* was given to us by Dr. C. Barigozzi of the Dept. of Genetics, University of Milano. His cultures had been established from an adult female obtained from wintering eggs, collected at the Comacchio salt ponds near Venice, and were maintained in Flemming's synthetic sea water with *Dunaliella tertiolecta* and Fleischmann yeast as food (Ballardin and Metalli, 1963). Under optimal agnotobiotic conditions adult females released a new brood of viviparous nauplii every four days.

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Dr. Barigozzi also sent a few wintering eggs; occasionally this viviparous strain of *Artemia salina* deposits durable eggs.

Axenization trials

1. Wintering eggs

The techniques of Gibor (1956a) and Provasoli *et al.* (1959b) needed minor modifications to wash free of contaminants the wintering eggs of the many strains of *Artemia.*

Unfortunately, the available wintering eggs of the Comacchio strain were so few that we could not wash them by the usual flotation method. These eggs were washed by serial transfers in sterile Pyrex spot-test slides: 9 baths (1 ml.) in sterile sea water followed by 3 baths in 1:1000 Merthiolate solution (lasting a total of 10 minutes) and 3 baths in sea water. They then were allowed to hatch; each hatched nauplius was in turn also washed repeatedly. In spite of the added precautions all nauplii were bacterized. The Comacchio eggs, unlike those of other strains, were heavily coated with detritus and, most likely, bacteria were not dislodged by our wash method.

2. Nauplii

Extensive serial washing of the viviparous nauplii collected from crude cultures in our laboratory failed to eliminate the bacteria, probably because the gut microflora remained untouched. With the hope that ready-to-hatch nauplii still inside the ovisac might be aseptic, a female with an ovisac was passed through several baths of Merthiolate 1:1000 solution and 75% alcohol. The artemia died, and the ovisac was opened in sterile sea water with glass needles under a germicidal hood. None of the nauplii recovered alive were bacteria-free.

3. Antibiotic tolerance

Because of the previous failures it became desirable to determine the tolerance of the adults and the nauplii to various antibiotics. Both stages had similar tolerances (Table I).

The sensitivity of the bacterial flora was tested with sensitivity discs (Difco). No single antibiotic completely inhibited bacterial growth. Several mixtures of the most effective antibiotics were tried (Table II).

Purification technique

Antibiotic mix 2 (Table II) in the wash solutions permitted elimination of all bacteria, but a *Saccharomyces* persisted. Fungicides could not be used because they were more toxic to *Artemia* than to the yeast. The yeast was ultimately eliminated by growing the *Artemia* in high-salinity media; details follow.

Elimination of bacteria. Synthetic salt solutions of different salinities were made from appropriate dilution of the stock salt solution mix MGSL (Table III). Unless otherwise indicated, growth media were also prepared from this stock salt mixture.

TABLE I

Tolerance to antibiotics of Artemia adults and nauplii
(μg./ml.)

Antibiotics	Safe[1]	Slight[2] inhibition	Marked[3] inhibition	Toxic[4]
Penicillin*	1850	3300	9900	
Streptomycin	200	300	400	1000
Polymixin*	228	338	435	456
Chloramphenicol	25	50	56	224 (Ab)
Naramycin	10	15	25	50
Kanamycin	250	500	750	
Neomycin	5	10	15	20 (Ab)
Nalidixin	50	100	150	200
Tetracycline	5	7.5	9	15 (Ab)
Trichomycin		50	75	100 (Ab)
Fungicides:				
Candicidin	2.5	3	5	7.5
Nystatin	10	15	30	
5,6-Dichlorobenzoxozaline**				0.1 ml.***
Lauryl hydroxamate**				0.1 ml.***
Methenamine mandelate	500	1000	2000	3000

* Units/ml.: pencillin 1 mg. = 1650 units; Polymixin 1 mg. = 7760 units.
** Obtained from Geigy Co.
*** ml. of saturated solution/ml. of medium.
Ab = Algae bleach within 24 hours.
(1) No apparent effect to *Artemia* adults and nauplii after 37 days.
(2) Slight inhibition of growth; animals recovered readily if transferred to *no* antibiotics after first signs of inhibition (7–30 days).
(3) Marked inhibition of growth; generally adults did not recover, nauplii often recovered but did not reach adulthood.
(4) Death occurs in 1–24 hours. Nauplii seemed more sensitive.

TABLE II

Antibiotic mixes
(μg./ml.)

Antibiotics and fungicides	Mix 2** 1 ml. =	Mix 2M** 1 ml. =	Mix 3*** 1 ml. =
Penicillin*	16500	16500	
Streptomycin	1000	1000	
Polymixin*	2880	2880	
Chloramphenicol	250	250	250
Candicidin		25	
Methenamine mandelate		1000	
Trichomycin			1000
Tetracycline			100
Neomycin			100

* Units/ml.: penicillin 1 mg. = 1650 units; Polymixin 1 mg. = 7760 units.
** Used at 0.1 ml./ml. for adult *Artemia*; 0.05 ml./ml. for nauplii.
*** Used at 0.1 ml./ml. to wash free of algae nauplii intended for nutritional experiments. *Artemia* nauplii must not be exposed to this mixture any longer than 6 hours; the nauplii were more sensitive than later stages.

A female with eggs in the ovisac was transferred to 30 ml. of 3 g.% salt solution to which was added 0.1 ml./ml. of antibiotic mix 2 and *D. tertiolecta* as the food organism. The female was transferred every hour during the working day to similarly prepared fresh media. This effectively prevented accumulation of fecal matter and bacteria. On the second or third day each nauplius as it emerged from the ovisac, was washed through 10 successive baths (30 minutes stay in each) of 10 ml. of the same salt-solution but with only 0.05 ml./ml. of antibiotic mix 2 and no food organisms. The nauplii do not feed for the first few hours. After the last wash *D. tertiolecta* was added; 24 hours later the nauplii were washed free of antibiotics and food organisms, and transferred into STP (Provasoli *et al.*, 1959a) for ½ hour to test for sterility. They were then placed into the maintenance medium MSWE or in MGSL (at 3% total salts) enriched with algal nutrients,

TABLE III

*MGSL stock salt solution**
g.% (w/v)

NaCl	17.30	
KCl	0.64	
$MgSO_4 \cdot 7H_2O$	3.40	
$MgCl_2 \cdot 6H_2O$	1.91	
$CaCl_2$	0.09	
$Na_2CO_3 \cdot H_2O$	0.03	
	23.37	
	2.76	H_2O of hydration
	20.61	g.% total salts less H_2O of hydration.

Dilutions (v/v) of this stock salt solution may be autoclaved without precipation.
The pH after autoclaving fluctuated between 7.0 and 7.8, well within the tolerance of the *Artemia* and the algae.
* MGSLE = MGSL + E enrichment (Table IV, note 2).
MGSLG = 50 mg.% glycylglycine are added as pH buffer.

and food organisms added. Ten larvae so washed proved bacteria-free. A *Saccharomyces* was detected a month or so later (STP is not a particularly good medium for yeasts).

Elimination of the yeast. Since, as noted, artemia nauplii and adults proved extremely sensitive to fungicides (Table I), we exploited the adaptability of the crustacean to high salinities. Surprisingly, the adult female could not tolerate sudden shifts in salinity. Gravid females when transferred from a 3% to a 6% salt solution invariably dropped the immature eggs and soon died. Nauplii, on the contrary, survived sudden salinity shifts: they could be transferred directly from a 3% to any concentration in the range 0.5–20.5% salt solution. Survival of the nauplii varied with age: 80% of the 6-hour-old nauplii survived direct transfer into 0.5–9% salts, and 35% when transferred into 12–20.5% salts; after the first naupliar molts, sensitivity to salinity changes increased and percentage survival decreased.

Use of a wide range of salinities (0.5–20.5%) entailed change in food organism:

D. tertiolecta did not grow at salt concentrations > 6%. Several algae were tested for ability to grow at the full range of concentrations after adaptation by serial transfer in gradually more diluted or concentrated solutions until the algae grew almost equally well at all concentrations.

Stephanoptera gracilis did not grow well < 6%. *Dunaliella parva, D. salina,* and *D. viridis* grew at all salinities, but *D. parva* bleached readily; *D. viridis* was acceptable to adult *Artemia* and not to the nauplii, while *D. salina* was acceptable to the nauplii as well as the adults, but grew slowly and was grazed too rapidly by the developing *Artemia.* Therefore, a mixture of *D. salina* and *D. viridis* was used.

Viviparous nauplii produced in MSWE (Provasoli *et al.*, 1959b, p. 254) by a bacteria-free but yeast-contaminated Comacchio female were transferred and grown to adulthood at the different salinities. Best growth was obtained in the 9% media. Viviparous nauplii released at this salinity were washed 10 times in 10 ml. of 9% media containing 0.5 ml./10 ml. of antibiotic mix 2M. Several nauplii proved free of bacteria and yeast. These were used to start the stock line which has been kept for over 3 years.

Maintenance of Stock Cultures

Several cumbersome trials led to the following two simple procedures for maintaining axenic cultures.

(1) Equal quantities (0.5 ml.) of a full-grown culture of *D. viridis* and *D. salina* were inoculated in screw-capped tubes (20 × 125 mm. Pyrex) containing 10 ml. of an autoclaved sea water enriched with organics (MSWE medium). After 10–15 days growth at 20–26° C. and *ca.* 200 ft. c., the algae cultures were very dense. One washed, newborn nauplius was inoculated in each of 10 tubes. Twenty to 25 days later, 3–5 of the 10 nauplii had become fertile females. The nauplii released were collected and the process repeated to carry on the new generations; any extra nauplii were used to carry on other experiments.

(2) The method was further simplified: 0.5 ml. of a dense suspension of the 2 algae and three washed new-born nauplii were inoculated simultaneously into each of a set of 5 tubes of STP. After 8–10 days at 20–26° C. and 200 ft. c., one of the three nauplii in each tube invariably outgrew the others. The two slow-growing artemia were removed, the other was left in the tube. Within 10 more days it developed into a fertile female; 5–6 days later the first batch of viviparous nauplii was released.

The nauplii taken from the parent's tube were washed three times in 10 ml. each of STP, the third wash tube was incubated in the dark, serving as a sterility test. Most of the algae were eliminated in the first two washes; therefore in the third tube the bacteria—if present—became evident (turbidity) before the algae masked them.

Several algae which had proved to be excellent food organisms for the Utah strain were also tested with the Comacchio strain, in the hope that some of them might be better for maintaining the bacteria-free stocks.

The algae were inoculated into STP, allowed to grow for 15 days, and then the nauplii were added. *Phaeodactylum tricornutum,* like *Dunaliella viridis,* was not acceptable to nauplii and early metanauplii. The diatom may have been too big to be ingested or it may have been indigestible. However, it was an excellent food

organism for the older and larger metanauplii and the adults. *Nitzschia acicularis* grew abundantly but growth of the artemia was slow (from nauplii to adults 40–50 days). A marine *Chlorella* sp. (No. 580, Guillard) allowed better growth—nauplii to adult in 30 days—but it was very difficult to wash the nauplii free from them; antibiotic mix 3 was not effective against it. *Rhodomonas lens* was an excellent food organism (fertile female in 18 to 20 days) but it grew slowly and was quickly and completely grazed: suspensions of the algae had to be added to the culture tubes every few days—an impractical procedure. *Isochrysis galbana* was also a good food organism but it was too sensitive to laboratory temperatures (20–28° C.).

Dunaliella salina + *D. viridis* remained best; *D. viridis* + *P. tricornutum* was also adequate but such cultures had to be watched closely. If the *P. tricornutum* dominated the system, growth of artemia equalled or bettered that obtained with the combination *D. salina* + *D. viridis*; if *D. viridis* dominated, *P. tricornutum* began to bleach and the *Artemia* died.

Trials with Artificial Media

Selective elimination of algal food. For nutritional experiments in artificial media, the viviparous parthenogenetic animals grown on live food had to be algae-free. Prolonged and tedious serial washes, spanning two or more days, were used at first. Fortunately, during the tolerance tests of the Comacchio strain to the antibiotics, tetracycline, trichomycin and chloramphenicol proved particularly inhibitory to *D. viridis* and *D. salina* and so served to eliminate the algae.

A brood of the Comacchio strain (30–60 nauplii preferably < 6 hours old) was collected by Pasteur pipette and transferred through 10 consecutive washes to sterile sea water + 1 ml./10 of antibiotic mix No. 3 (Table II). The nauplii were left in each wash 20 minutes, the whole operation lasting four hours. This procedure inhibited the algae without injuring the nauplii. If the nauplii had already begun feeding (8 or more hours old) the gut appeared green; in such cases after every 3 baths in sea water, the nauplii were transferred to, and left for 30 minutes, in a tube containing, in addition to the antibiotic mix 3, particulate starch (dry insoluble rice starch, autoclaved, suspended in H_2O, added dropwise). *Ad libitum* ingestion of the particulate starch induced defecation, thus cleaning the gut of surviving *D. viridis*. These manipulations were made in a transfer hood under a germicidal UV lamp.

Growth in artificial media. Several artificial media used to grow the axenic Utah strain of *Artemia* from nauplii to fertile females and egg deposition were tested with the Comacchio strain. The media were composed of (1) a liquid phase containing mineral salts, amino acids, liver extract, hydrolyzed DNA and RNA, eight vitamins, cholesterol, and (2) a fine particulate phase composed of precipitated egg albumin and rice starch. These media proved inadequate for the Comacchio strain. Nauplii (*ca.* 0.9 mm.) developed in 20–30 days into big metanauplii (*ca.* 3.5 mm., ½ the size of an early adult) which did not grow any further and died. If early metanauplii were inoculated in the artificial media they developed into young (5–7 mm.); if young adults were inoculated, they developed into infertile females (7–10 mm.). Young females fed with these artificial media were able to produce 1–2 normal viviparous broods (30–120 viable nauplii) but

died soon after. Variations of the components of the media did not substantially improve growth. Evidently the Comacchio strain needed additional growth factors.

Effects of Salinity and Enrichments on the Growth of Artemia

Purification of the Comacchio strain coincided with preliminary experiments on the Utah strain to determine the effects of salinity on egg production (D'Agostino, 1965).

At first the food algae for the Utah strain (a mixture of *D. tertiolecta, D. parva,* and *D. primolecta*) were grown on agar slants, the algae were scraped off, suspended in sterile sea water, centrifuged, and added as slurry *ad libitum* (as they were consumed by the growing *Artemia*) in the various dilutions of MSGL (0.5–20.5% salt concentrations).

Unexpectedly, even though the algae were abundant, only infertile Utah adults were obtained at salt concentrations < 3%; some nauplii were produced at 3%; and durable eggs were obtained only at concentrations > 6% (Table IV). In a later experiment the conditions were fortuitously changed: (a) the MGSL base was enriched with E (Table IV, note 2) to favor algal growth; (b) having

Table IV

Effect of enrichments and salinities on A. salina

Artemia strain / % Total salts	Utah Strain (Great Salt Lake)						Comacchio Strain (Italy)					
	Inorganic enrich. (1)			Organic enrich. (2)			Inorganic enrich. (3)			Organic enrich. (4)		
	Stage reached	Nauplii (5)	Eggs (6)	Stage reached	Nauplii	Eggs	Stage reached	Nauplii	Eggs	Stage reached	Nauplii	Eggs
0.5	Y-♀	0	0	♀	0.3	6.67	J	0	0	♀	5.0	0
1	♀	0	0	♀	1.3	6.8	Y	0	0	♀	5.4	0
3	♀	2.5	0	♀	0.8	4.73	♀	0	0	♀	0.2	0
6	♀	3.6	2.4	♀	1.8	11.20	♀	4.8	1.0	♀	1.9	0
9	♀	4.0	2.1	♀	3.0	5.69	♀	4.3	4.0	♀	6.3	2.9
12	♀	4.6	2.2	♀	1.9	3.63	♀	1.8	2.1	♀	5.8	2.4
15				♀	5.0	4.37	♀	4.7	3.1	♀	6.0	0.9
18				♀	3.1	2.2	♀	4.9	3.2	♀	5.3	2.1
20.5				♀	1.3	0.66	♀	7.9	2.3	♀	3.0	1.7

(1) Base = MGSL dilutions, no enrichment; various species of *Dunaliella*, grown on inorganic agar slants added *ad libitum*. One experiment.

(2) Base = dilutions of MGSL enriched with 5 ml./100 of STP (see text); or with 5 ml./100 STP + 2.5 ml./100 ES; or with 1 ml./100 E + 5 ml./100 STP. One ml./100 E introduces in percentage final medium; $NaNO_3$ 30 mg.; Na_2 glycerophosphate 5 mg.; UII metals 0.1 ml.; vitamins 8a 0.1 ml.; B_{12} 0.1 μg.; Fe (as EDTA) 50 μg. (see notes for Table V). Average of these 3 experiments.

(3) Base = dilutions of MGSLG (see note Table III) enriched with 5 ml./100 of ES or with 2.5 ml./100 ES + 1 ml./100 E_2. Average of these 2 experiments.

(4) Base = dilutions of MGSLGE (see note Table III) enriched with 1 ml./100 of E_1. One experiment.

(5) Viable nauplii produced per female/days of female life.

(6) Durable eggs produced per female/days of female life.

exhausted the supply of algae grown on slants just when most Utah artemias had reached the adult stage, algae grown in STP liquid medium were added unwashed, thus introducing with the algae about 0.5–1 ml. of STP per 10 ml. of base. These changes induced egg deposition at all salt concentrations even in the range 0.5–3% (Table IV), indicating that the organic enrichment affected egg production at low salinities. This effect was similar to that observed with *Tigriopus* (Shiraishi and Provasoli, 1959), the food algae, grown on a medium already containing an organic enrichment (5 ml./100 of STP), became an insufficient food source after several aseptic generations of *Tigriopus*; at the time the nutritional insufficiency was corrected by the addition of a vitamin mixture to the medium in which the algae and *Tigriopus* were growing.

TABLE V

Enrichments

(% in final medium when used at stated addition)

Enrichment designation	STP	E_2	ES	E_2	ES+STP	E_1	STP
Components \ addition	5 ml./100	1 ml./100	5 ml./100	1 ml./100	2.5 ml./100 each	1 ml./100	100%
Soil extract	0.25 ml.				0.125 ml.		5 ml.
$NaNO_3$	1 mg.	5 mg.	17 mg.	5 mg.	9 mg.	25 mg.	20 mg.
K_2HPO_4	0.05 mg.				25 μg.		1 mg.
Na_2 glycerophosphate		2 mg.	2.5 mg.	2 mg.	1.25 mg.	10 mg.	
$Na_2SiO_3 \cdot 9H_2O$						5 mg.	
Metals UII[1]				0.01 ml.		0.05 ml.	
Metals PII[2]			1.25 ml.	0.05 ml.	0.62 ml.	0.12 ml.	
Metals SII[3]				0.05 ml.		0.12 ml.	
Fe (as EDTA)[4]			0.125 mg.	5 μg.	62 μg.	50 μg.	
Vitamins 8a[5]	0.005 ml.				0.0025 ml.	0.05 ml.	0.1 ml.
Thiamine			25 μg.		12 μg.		
Biotin			0.25 μg.		0.12 μg.		
B_{12}			0.5 μg.		0.25 μg.		
Sucrose (s) or glucose (g)	5 mg. (s)				2.5 mg. (s)	25 mg. (g)	100 mg. (s)
NaH-glutamate	2.5 mg.			5 mg.	1.25 mg.		50 mg.
DL-alanine	0.5 mg.			1 mg.	0.25 mg.		10 mg.
Glycine	0.5 mg.			1 mg.	0.25 mg.		10 mg.
Glycylglycine						25 mg.	
Yeast autolysate (A) or extract (E)	1 mg. (A)				0.5 mg. (A)	0.5 mg. (E)	20 mg. (A)
Trypticase	1 mg.				0.5 mg.		20 mg.
Liver Oxoid L.25						0.5 mg.	
Urea						1 mg.	
Tris buffer			25 mg.		12.5 mg.		

[1] Metals U II (Aldrich & Wilson 1960) 1 ml. contains: Na_2 EDTA 0.15 mg.; $FeCl_2 \cdot 4H_2O$ 1 μg.; $CuCl_2$ 1 μg.; $MnCl_2 \cdot 4H_2O$ 10 μg.; $ZnCl_2$ 5 μg.; $NiCl_2 \cdot 6H_2O$ 1 μg.; $AlCl_3 \cdot 6H_2O$ 5 μg.; $CoCl_2 \cdot 6H_2O$ 5 μg.; RbCl 10 μg.; $BaCl_2$ 1 μg.; H_2SeO_3 5 μg.; NH_4VO_3 1 μg.; TiO_2 5 μg.; $ZrOCl_2 \cdot 8H_2O$ 5 μg.; $K_2Cr_2O_7$ 5 μg.; $SrCl_2 \cdot 6H_2O$ 5 μg.; $Na_2MoO_4 \cdot 2H_2O$ 5 μg.; H_3BO_3 5μg.; CsCl 5 μg.; $(NH_4)_2$ Ce $(NO_3)_6$ 1μg.; $CdCl_2 \cdot 2\frac{1}{2}$ H_2O 1μg.; $SnCl_2 \cdot 2H_2O$ 1μg.; $RuCl_3$ 1 μg.; $RhCl_3$ 1 μg.

[2] [3] [5] See Provasoli *et al.* (1957).

[4] Dissolve 351 mg. of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 330 mg. Na_2EDTA in 500 ml. H_2O; 1 ml. of this solution = 0.1 mg. Fe.

Various enrichments (Table V) were formulated and tried on the Utah and Comacchio strains employing the *Tigriopus* technique, *i.e.*, the algae and the *Artemia* nauplii were inoculated simultaneously in the enriched media. Salinity-adapted *D. salina* and *D. viridis* were employed as food organisms. The effect on egg deposition at low salinities induced by the combination E + STP was confirmed with this technique for the Utah strain with the *D. salina* + *D. viridis* food combination (Table IV).

Similar experiments were done with the Comacchio strain. Table VI shows the effect of different enrichments and different salt concentrations after 26 days

growth. *D. salina* was inoculated in duplicate sets of tubes and allowed to grow 10 days; the density of the algal growth was similar in all enrichments and salinities. Single algal-free washed nauplii were inoculated in each tube and the experiment read at the 26th day, when the algae had been grazed almost completely in some tubes while much algae still remained in other tubes. As evident, the effect of low salinities was even more pronounced in the Comacchio strain: concentrations $< 3\%$ slowed development; this effect was gradually overcome as the additions became richer in organic nutrients, E_1 being the most satisfactory enrichment.

Experiments in which the algae were seldom limiting (weekends) were done by transferring the Comacchio artemias to a new tube of algae whenever the previous tube became depleted of algae due to grazing (Table IV, columns 3, 4). Salinities below sea water concentration with inorganic enrichment not only slowed growth but allowed growth only to juvenile or young stages, with no further development even though food was abundant and the animals remained alive for

TABLE VI

Effect of salinity and enrichments on the Comacchio strain[1]

Enrichment \ % Total salts	0.5%	[3]	1 g.%	[3]	3 g.%	[3]	6 g.%	[3]
STP 5 ml./100	IV medium[2]	+++	Juvenile	+	IV big	+	IV big	+
E_2 1 ml./100	IV medium	+	Juvenile	+	Young	±	Young	±
E_2 1 ml./100	IV medium	+++	Young	−	Young	−	Young	±
STP 5 ml.	Young	+++	Young ♀	+	♀	−	♀	++
+ ES 5 ml./100								
E_1 1 ml./100	♀	−	♀	+	♀	+	♀	±

[1] 26 days growth; basal medium = dilutions of MGSL.

[2] For description of morphological appearance see Provasoli and Shiraishi (1959b). The length in mm. of the various stages follows: IV medium, 2.5–3.5; IV big, 4–6; juvenile, 6–8; young, 8–9; young female, 9–12; fertile females, 12–14.

[3] Density of algae at 26 days: +++ very many; ++ and + many; ± few; − scarce.

10–20 days. The organic enrichment E_1 overcame the low-salinity effect and allowed growth to adult fertile viviparous females.

EFFECTS OF SALINITY AND FOOD AVAILABILITY ON OVO- AND VIVIPARITY

The two strains of *Artemia* differ in reproduction. The Utah strain is amphigonic and after copulation may deposit several types of eggs in the laboratory: (a) green or white eggs which fail to hatch; (b) pinkish eggs which may hatch 24–48 hours after deposition; (c) hard-shelled pale or dark-brown eggs equivalent to the wintering (or durable) eggs found in salinas and salt lakes; such eggs when deposited are in diapause and require rehydration for hatching.

The Comacchio strain is parthenogenetic oviparous and viviparous; usually the eggs hatch in the ovisac and the nauplii emerge. The organic enrichment at salt concentrations $\leqq 3\%$ predominately induces wintering eggs in the Utah strain; the Comacchio strain is exclusively viviparous. Interestingly, wintering eggs were produced by the Comacchio strain at salt concentrations $\geqq 9\%$. The results in Table IV are expressed as numbers of nauplii or durable eggs per day of female

life and are the mean of several experiments. Most females lived 30 ± 5 days; one lived 10 days and four 37–40 days.

Both strains of *Artemia* grazed the algae more vigorously at higher salinities; therefore the adults had to be transferred more often to new tubes preinoculated with algae to try to keep them fed *ad libitum*. Production of nauplii or wintering eggs varied more widely in higher salinities; this was correlated with periods of less available food, *i.e.*, during weekends. To investigate this possibility, the Comacchio strain was selected because, being parthenogenetic, only one animal—the female—was needed, making it easier to maintain a more constant supply of algal food under aseptic conditions. Mature females were transferred singly in tubes rich in algae. In 6–7 days they produced a viviparous brood of nauplii; by this time the algae had almost completely disappeared. The nauplii were taken out of the tube and the female was starved; 5–7 days later durable eggs were produced. The female was now transferred to a tube rich in algae and in 5–7 days produced again a viviparous brood. This experiment was repeated several times in MGSL base at 5% + E_1 enrichment or in a commercial sea water-type salt mix (RILA, same concentration) + E_2 + STP.

Ballardin and Metalli (1963), working with bacterized cultures fed with a "marine *Chlaymdomonas*" (supplied by us = *Dunaliella tertiolecta*) observed that under their conditions (artificial sea water + organic enrichment) the Comacchio strain tended to deposit wintering eggs. They found no correlation between oviparity and type of food, overcrowding, higher salinities, temperature, and illumination. It is not clear whether they subjected the Comacchio strain to deliberate starvation.

"Hypo-alimentation" of the S. Gilla (Cagliari) strain, from larval stages to the 1st deposition gave viviparous nauplii, but if scarcity of food continued, the 2nd deposition was almost completely oviparous. They concluded that oviparity in the Cagliari strain is influenced by the length of hypo-alimentation. Their culture conditions were different from ours; they achieved hypo-alimentation by less frequent additions of algae, thus causing slower growth and higher mortalities. We starved full-grown and fertile females, only during the egg production-deposition period.

Discussion

Experiments on *Tigriopus* and *Artemia* had shown that the nutritional value of different species of algae varied widely even among species of the same genus: only four algae of the 20 tried were a complete food for at least one generation of both Crustacea. With *Tigriopus* (whose eggs hatch immediately), only one species of algae, *Monochrysis lutheri*, was a complete food for more than 20 generations of the crustacean. Nutritional deficiencies developed after 1–9 generations of *Tigriopus*, depending on the species of algal food, with gradual reduction in size of adults and subsequent infertility. With two species of algae, *Isochrysis* and *Chroomonas*, normal growth and fertility of *Tigriopus* was restored by adding a vitamin mixture or glutathione. At the time, speculations were made as to whether the vitamins were absorbed directly by *Tigriopus* or were taken up by the algae, and perhaps had affected algal metabolism in some way (Shiraishi and Provasoli, 1959).

In the experiments with the two strains of *Artemia* the nutritional deficiencies were most evident when the organisms were subjected to the physiological stress of low salt concentrations. The possibilities are: has the osmotic stress affected mainly *Artemia* (*i.e.*, by changing its nutritional requirements) or mainly the algae (by changing the algal metabolism, hence their nutritional value) or has it affected both—prey and predator? Strangely, as in *Tigriopus*, an organic supplement restored speed of development and fertility. Again the question remains: do organic substances act directly on the *Artemia* or *via* the food organisms?

New circumstantial evidence makes unlikely the hypothesis of the direct effect of organic nutrients on *Tigriopus* and *Artemia*. Stephens and Schinske (1961) found that most soft-bodied invertebrates can absorb and incorporate from dilute solution radioactive amino acids and sugars. The only invertebrates which had negligible uptake were the Crustacea, probably due to the impervious chitinous exoskeleton. Our experience with the Utah strain of *Artemia* grown in artificial media supports the hypothesis that these animals depend on particulate organic matter as their main source of food, but that they also imbibe very small quantities of liquid.

(a) To obtain *Artemia* juveniles we need $\sim$ 10 mg.% of precipitated albumin and 50 mg.% of insoluble starch. We can replace partly the protein particles with various amino acid mixtures mimicking albumin, liver, etc., but about 300 mg.% of assorted amino acids are needed to replace 5 mg.% of precipitated albumin—hence solutes are taken up but have $\sim$ 1/60 the efficiency of particulates. It was impossible to replace the precipitated albumin completely because the amino-acid mixtures become inhibitory at or above 600 mg.% despite the notorious ability of *Artemia* to grow at $\sim$ 20 g.% total salts.

(b) Most of the vitamins needed by *Artemia* are water-soluble; they are apparently consumed by *Artemia* directly from the solutions and are not appreciably absorbed on the particles of the medium, even during sterilization, because the supernatant of the autoclaved vitamin-containing medium can support growth when added to the particulate of a non-vitamin-containing medium, and not the reverse. The levels of vitamins needed by the Utah strain are in the mg.% range for thiamine, nicotinic, and pantothenic acids and in the 0.1 mg.% range for riboflavin, pyridoxamine, and folic acid. The need for such high concentrations supports the hypothesis that marine Crustacea imbibe small quantities of liquid. Vitamins are generally needed in minute quantities (1–100 μg.%) but apparently are not toxic at several 100-fold concentrations.

Tigriopus can be grown to the second copepodite stage in artificial media. The media are similar to the ones for *Artemia*, differing mainly in the ratios of the various components. The vitamin mixture found adequate for *Tigriopus* in artificial media is extremely concentrated when compared with the vitamin mixture employed to restore fertility of *Tigriopus* grown on *Isochrysis* or *Chroomonas* (Table VII). Hence direct uptake of vitamins by *Tigriopus* is improbable.

These data seem to exclude the possibility that the remarkable effect of organic substances on the two *Artemia* strains at low salinities can be ascribed to the direct absorption by the artemias of 25 mg.% glucose + 1 mg.% urea, and 0.5 mg.% each of yeast extract and Oxoid liver. The effect may be mediated through the algae.

The *Dunaliella* species so far studied require for growth neither vitamins nor organic compounds of P and N: they are photoautotrophic. However, growth of *D. salina* and *D. viridis* is stimulated by yeast extract, brain-heart infusion, and glucose; some amino acids serve efficiently as N sources. *D. viridis* seems more heterotrophic than *D. salina,* though heterotrophy does not sustain growth in darkness of either (Gibor, 1956b). Since organic substances are actively taken up by these two algae, it is possible that they might be concentrated in the algal cells, enriching their value as food, or may have affected the metabolism and chemical composition of the algae. The ability of many algae to concentrate trace metals (Black and Mitchell, 1952), phosphorus (P-luxury consumption) and other nutrients from dilute solutions is well known. Spoehr and Milner (1949) have shown that *Chlorella pyrenoidosa* grown in N-deficient media are low in proteins and rich in fats, the opposite occurs in normal (N-rich) media; changes in cell composition

TABLE VII

Vitamin mixtures for Tigriopus
(μg. per 100 ml. of final medium)

	Supplement for mono-axenic cultures	For artificial media
Thiamine	61	750
Nicotinamide	6	300
Ca pantothenate	6	375
Riboflavin	0.3	22.5
Pyridoxine	80	150
Folic acid	0.4	52.5
Biotin	4.6	7.5
Putrescine		302
Inositol	9	75
Folinic acid	0.4	

were induced in other algae by nutritional and other cultural conditions (Fogg and Than-Tun, 1960).

Since the nutritional deficiencies of *Artemia* were evident at salinities below sea water concentration, one wonders whether osmotic stress may have affected directly the algae or the Crustacea or both. Unfortunately our experiments on the nutrition of *Artemia* in artificial media were done at sea water concentration and information is lacking on how salinities affect the nutritional requirements of *Artemia.*

It is known that algal photosynthetic rate and glycerol production are greatly affected by NaCl concentrations: twice as much radioactive C is fixed by *D. tertiolecta* at 14% NaCl than at 0.14% even though the alga had been preconditioned to different salinities by 5 weekly transfers. The quantity of glycerol produced at high salinity is 60 × higher, indicating that the nature of the photosynthate varies with salinity, *i.e.,* more glycerol is produced at high salinity and more polysaccharides at low salinities; but at low salinities C-fixation is twice as low (Craigie and McLachlan, 1964). In our cultures low-salinity stress may have similarly changed the metabolism and cell composition of the preconditioned algae.

Glycerol is an important metabolite in the physiology of the early stages of *Artemia.* Free glycerol synthesis from stored trehalose or glycogen follows re-

hydration, emergence and hatching of the *Artemia* nauplii. According to Clegg (1964) the amount of glycerol produced by the emerging nauplii increased with increased osmotic pressure of the medium. The glycerol is released into the medium following the hatching of the nauplii. This behavior parallels that of the *Dunaliella* (Craigie *et al.*, 1966). No studies have been done on the synthesis of glycerol by the adult *Artemia* in response to salinity stress.

Finally, osmotic stress may have affected the nutritional requirements of *Dunaliella*. Temperature stress (at lethal temperatures) has been shown to affect widely the nutritional requirements of *Ochromonas* (Hutner *et al.*, 1957), and *Gyrodinium cohnii* (Gold and Baren, 1966), inducing new or higher requirements in vitamins and trace metals. While temperature stress may stop growth of some algae in unenriched media, the growth of *Dunaliella* seemed unaffected or else affected in a minor way after these species were adapted to hyposalinity, *i.e.*, they grew about as well at all salinities. Nonetheless rich organic supplements, *e.g.*, yeast and liver extracts, were needed to restore normal development and fertility in *Artemia*. It seems, then, justified to postulate that hyposalinity may have affected primarily the physiology of the algae, and that the organic supplement somehow restored their nutritional value for *Artemia*.

SUMMARY

1. Two tetraploid strains of *Artemia salina* were axenized. The bisexual strain from the Great Salt Lake was obtained bacteria-free by serial washes of the wintering eggs in Merthiolate. The parthenogenetic strain from the Comacchio salines was axenized by serial washes of gravid females and nauplii in antibiotics; the yeast infection was eliminated by high salinity.

2. Methods for the maintenance of the bacteria-free stocks were defined. Various phytoflagellates were found suitable food organisms for both strains of *Artemia* when grown in appropriately enriched sea water.

3. Two species of *Dunaliella* were adapted to grow in synthetic salt solutions ranging from 0.5 to 20.5% total salts. These conditions permitted the study of the effect of salinity on egg production of *Artemia* in diaxenic culture.

4. Great Salt Lake *Artemia* grown at various salinities, in inorganic enrichments and fed *ad libitum* with salinity-adapted *D. viridis* and *D. salina*, failed to deposit eggs in solutions containing less than 3% salts. The Comacchio *Artemia* was even more sensitive: in low salinities development was inhibited even though the algae were abundant.

5. The reduced fecundity of *Artemia* at low salinities could be reversed by adding organic enrichments, *i.e.*, small quantities of glucose, liver extract, yeast extract and vitamins. The organic enrichment was thought to affect the metabolism of the algae rather than that of *Artemia*.

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